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Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/61334 A2

(54) Title: NEW METHODS FOR SURFACE MODIFICATION OF SILICA FOR USE IN CAPILLARY ZONE ELECTROPHORESIS AND CHROMATOGRAPHY

(57) Abstract: The present invention refers to the use of novel molecules able to bind tenaciously to silica, borosilicate and silicate surfaces, and thus to modify their properties and characteristics. When applied to silica-based chromatography, it offers important advantages in all cases in which it is necessary to modulate the interaction of analytes with the stationary phase. In capillary zone electrophoresis (CZE), such compounds will eliminate or invert the electroendoosmostic (EEO) flow, greatly simplifying the analysis of negatively-charged compounds and permitting the analysis of bio(macro)molecules via the direct use of naked capillaries.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
SCB611PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 01/ 01544	13/02/2001	18/02/2000
Applicant		
CITTERIO, Attilio		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

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   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

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2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

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6. The figure of the **drawings** to be published with the abstract is Figure No. –

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

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   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
/EP 01/01544

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N27/447

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C07D C08G

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 391 274 A (SHIEH CHIA-HUI) 21 February 1995 (1995-02-21) column 4, line 54 -column 6, line 52	1,5,7-9, 11-15
Y	DE 836 937 C (NORDMARK-WERKE GMBH) 17 April 1952 (1952-04-17) figure 1	1,5,7-9, 11-15
A	US 4 904 629 A (GALLA EDWARD A ET AL) 27 February 1990 (1990-02-27) column 2, line 25	3
A	US 2 417 992 A (VICTOR NIEDERL ET AL) 25 March 1947 (1947-03-25) claim 1	5

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
29 October 2001	08/11/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Duchatellier, M

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4 014 678 A (HUPPI GERHARD ET AL) 29 March 1977 (1977-03-29) abstract	5
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
[redacted]/EP 01/01544

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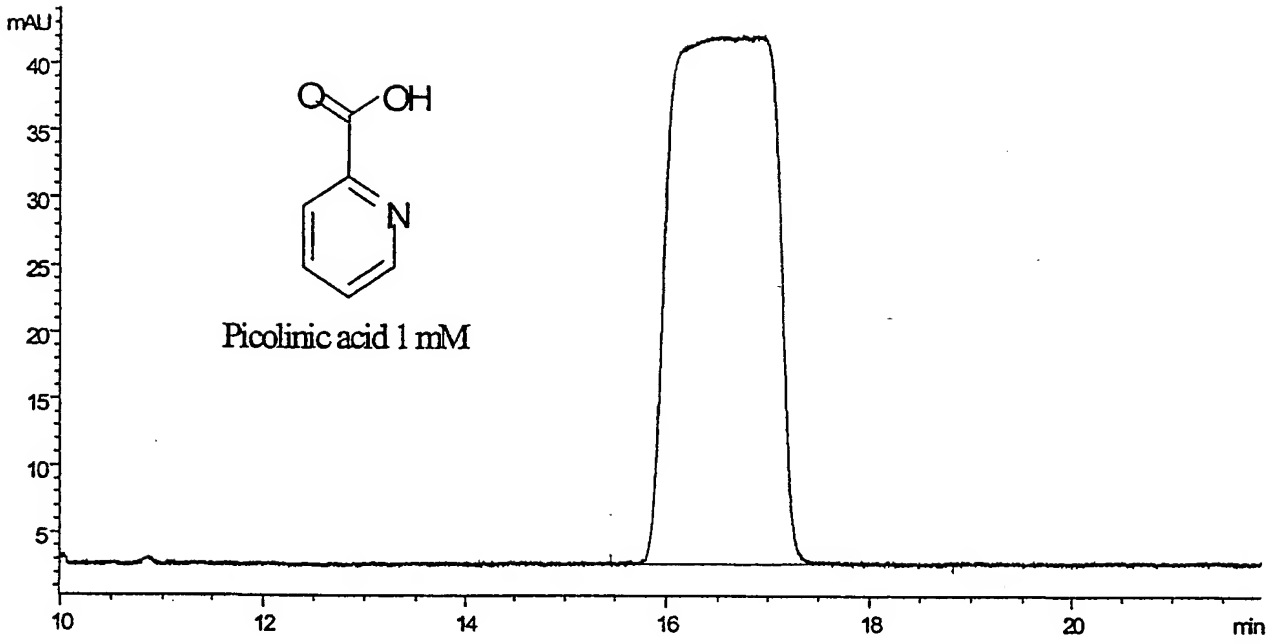


Figure 1: analysis conditions: fused silica capillary ϕ = 50 µm, $L_{tot.}$ = 60 cm, 50 mM borate buffer, pH = 9.0, + 15 kV, T = 20°C, λ = 210 nm.

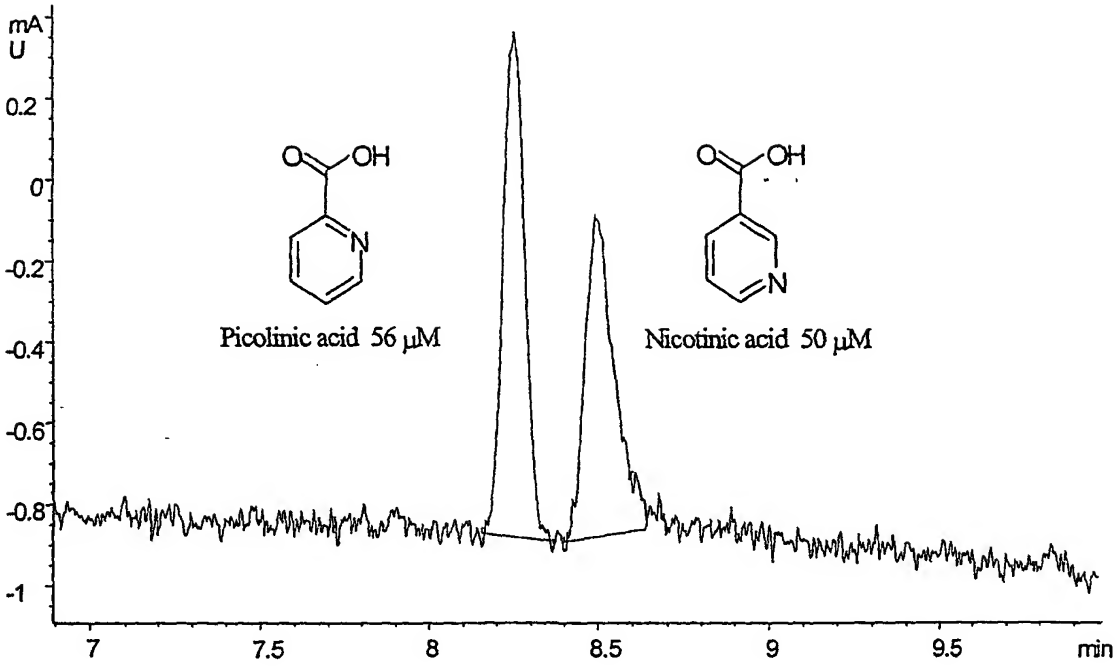


Figure 2: analysis conditions: fused silica capillary, pre-treated for 5 min with a 1 mM solution of compound (1), $\phi = 50$ µm, $L_{tot.} = 60$ cm, 25 mM borate buffer, pH = 9.0, -20 kV, T = 20°C, $\lambda = 210$ nm.

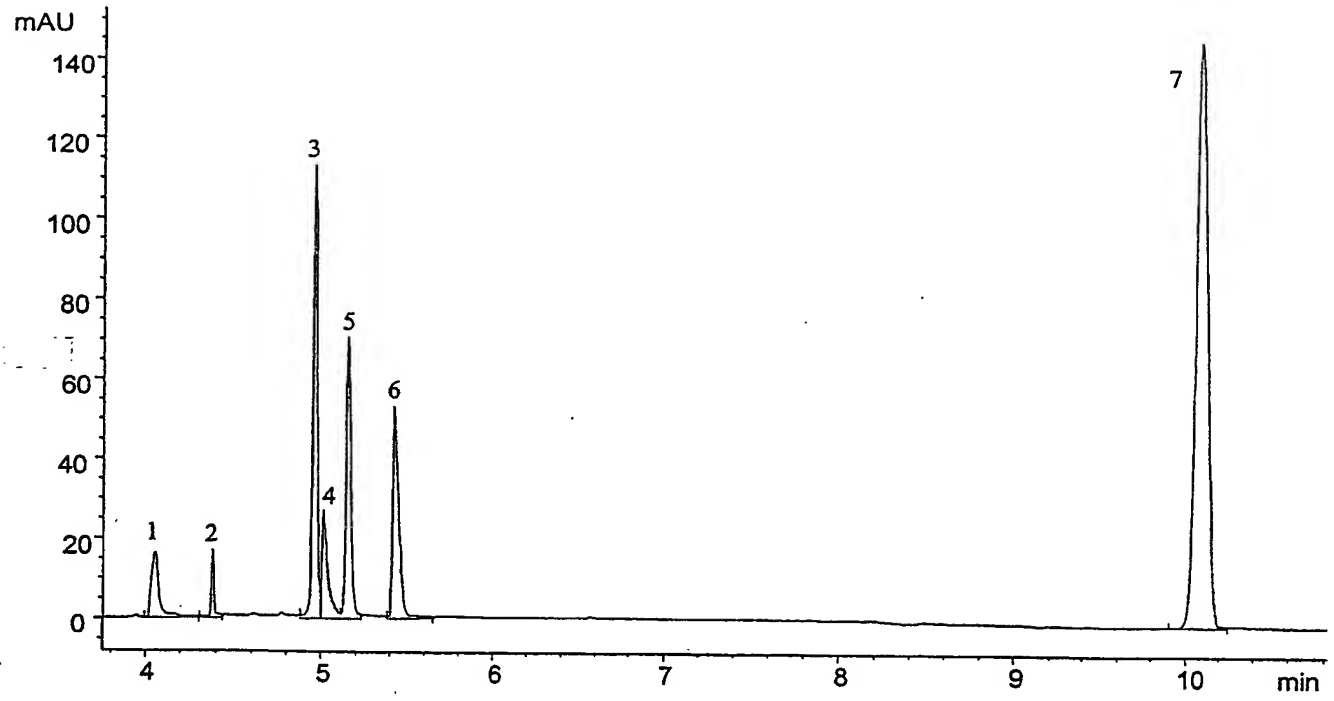


Figure 3: analysis conditions: fused silica capillary, ϕ = 50 µm, $L_{tot.}$ = 60 cm, 25 mM borate buffer, pH = 8.5, - 20 kV, T = 20°C, λ = 210 nm. Analyte concentration: 0.2 mg/ml.

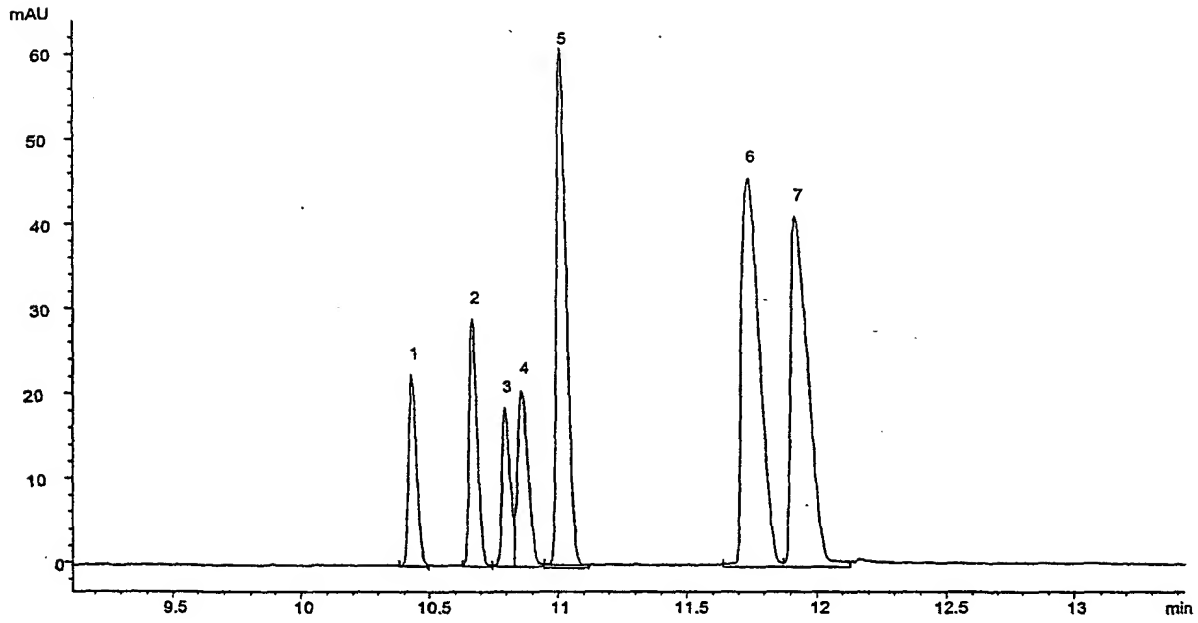
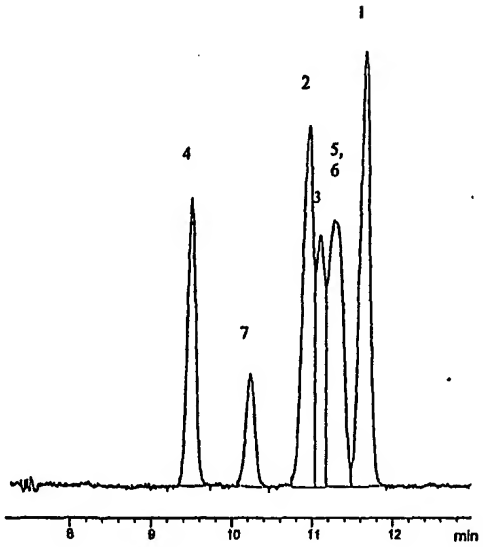


Figure 4: analysis conditions: fused silica capillary ϕ = 50 µm, $L_{tot.}$ = 100 cm, 25 mM borate buffer, pH = 8.5, - 25 kV, T = 25°C, λ = 210 nm. Analyte concentration: 0.14 mg/ml.



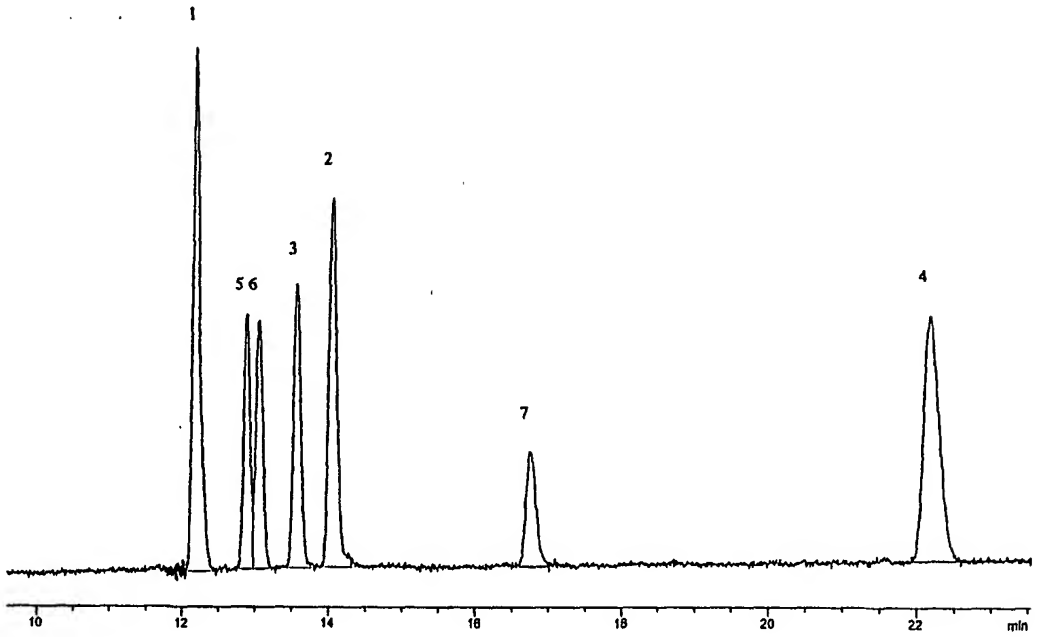


Figure 5A: analysis conditions: uncoated fused silica capillary $\phi = 50\ \mu m$, $L_{tot.} = 50$ cm, 25 mM borate buffer, pH = 9, + 15 kV, T = 25°C, $\lambda = 210$ nm.i

Figure 5B: analysis conditions: fused silica capillary $\phi = 50\ \mu m$, $L_{tot.} = 50$ cm, 25 mM borate buffer, pH = 9, - 25 kV, T = 25°C, $\lambda = 210$ nm.

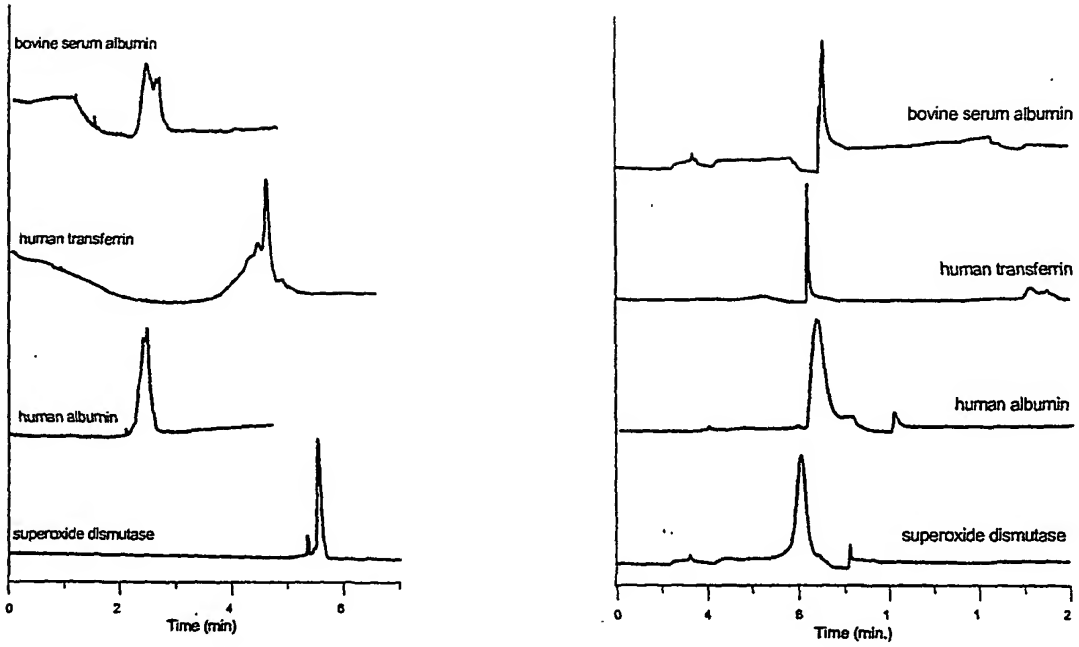


Figure 6: Separation of a number of protein markers, injected in a covalently coated (left) and in a **Q-PzI** treated (right) capillary, respectively. Capillary length 37 cm, 50 µm i.d.. Separation conditions were: run at 200 V/cm, sample injection by pressure for 2 sec, 5 psi/s, detection at 214 nm. In both cases the running buffer was 25 Mm Na tetraborate, Ph 9.0.

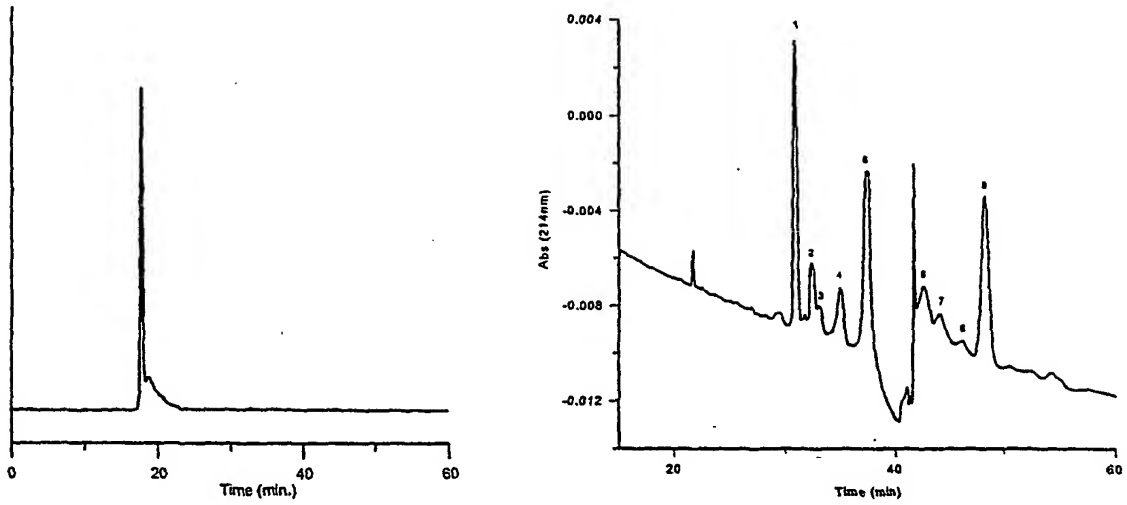


Figure 7: Separation of protein mixture with Pi ranging from Ph 3-10 (right) with **QpzI** treated capillary 77 cm long, 50 μm i.d.; (left) covalently coated capillary , 77 cm long, 50μ m, i.d..

Separation conditions: 250V/cm, sample injection by pressure for 5 sec, running in tetraborate buffer Ph 9.0.(1) Horse myoglobin, (2) bovine carbonicanhydrase B, (3) human carbonicanhydrase B, (4) β-lactoglobulin A, (5) soybean trypsin inhibitor, (6) lentil-lectin Pi 8.15 (7) lentil-lectin Pi 8.55, (8) lentil-lectin Pi 8.65, (9) trypsinogen

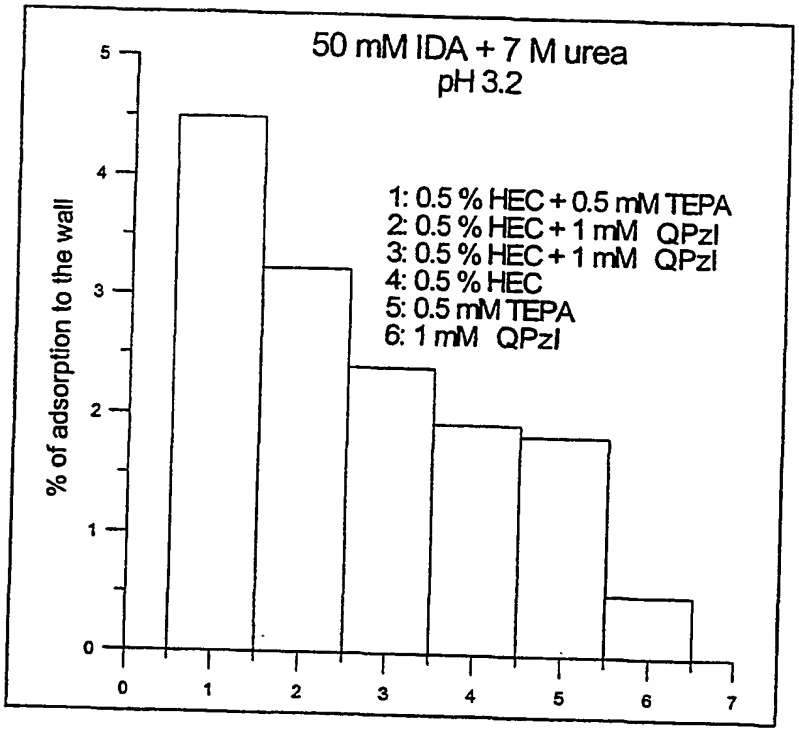


Figure 8: inhibition ability of different additives to the binding of proteins to the silica wall. The electrophoretic runs have been performed in 50 Mm IDA buffer, in presence of 8 M urea (apparent Ph of 3.2) in Waters Quanta 4000E instrument, in a 27-cm-long uncoated capillary, 50 μm ID. Sample: mixture of α and β human globin chains, 2 mg/Ml. After 10 consecutive runs, the adsorbed proteins are eluted electrophoretically in 25 Mm phosphate buffer, Ph 7, containing 60 Mm SDS and detected at 210 nm.

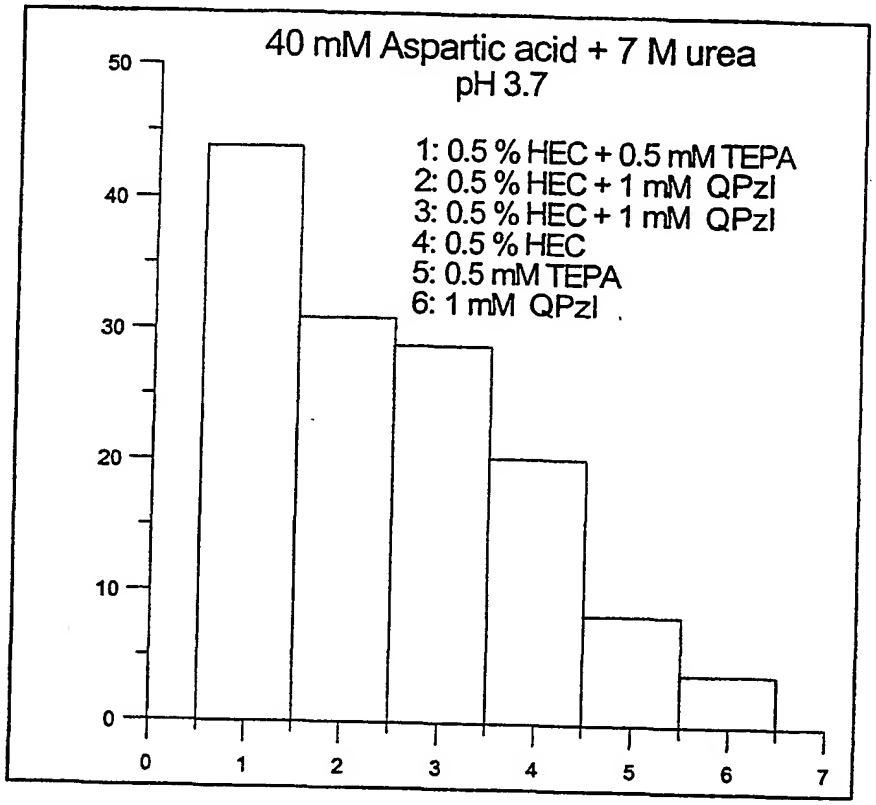


Figure 9: inhibition capability of various additives toward the adsorption of proteins to the silica wall. The electrophoretic runs have been executed in 50 mM Asp buffer in presence of 8 M urea (apparent pH 3.8) in a Waters Quanta 4000E instrument, in 27-cm-long, uncoated capillary, 50 μm ID. Buffer: a mixture of α e β human globin chains, 2 mg/mL. After 10 consecutive runs, the adsorbed proteins are eluted electrophoretically in 25 mM phosphate buffer, pH 7, containing 60 mM SDS and detected at 210 nm.

PCT

CONFIRMATION COPY

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

PCT/EP 01/01544
International Application No.

13 FEB 2001 (13.02.2001)
International Filing Date

EPO - Munich 60

EUROPEAN PATENT OFFICE
PCT INTERNATIONAL APPLICATION
15 Feb. 2001
Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* SCB611PCT

Box No. I TITLE OF INVENTION NEW METHODS FOR SURFACE MODIFICATION OF SILICA FOR USE IN CAPILLARY ZONE ELECTROPHORESIS AND CHROMATOGRAPHY

Box No. II APPLICANT

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Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

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[ ] inventor only *(If this check-box is marked, do not fill in below.)*

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[X] Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: [X] agent [ ] common representative

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This person is:
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- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: ~~Italy~~ IT

State *(that is, country)* of residence: ~~Italy~~ IT

This person is applicant for the purposes of:
- [X] all designated States
- [ ] all designated States except the United States of America
- [ ] the United States of America only
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Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: ~~Italy~~ IT

State *(that is, country)* of residence: ~~Italy~~ IT

This person is applicant for the purposes of:
- [X] all designated States
- [ ] all designated States except the United States of America
- [ ] the United States of America only
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Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- [ ] all designated States
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Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [ ] the United States of America only
- [ ] the States indicated in the Supplemental Box

- [ ] Further applicants and/or (further) inventors are indicated on another continuation sheet.

BOX V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

☒ **AP** **ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, MZ Mozambique, SD Sudan, SL Sierra Leone, SZ Swaziland, TZ United Republic of Tanzania, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT

☒ **EA** **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT

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Check-box reserved for designating States which have become party to the PCT after issuance of this sheet:

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Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 18 February 2000 (18.02.2000)	MI2000A 000294	~~Italy~~ IT		
item (2)				
item (3)				

☐ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): _____

** Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)* : ISA/

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets**:

request	: 04
description (excluding sequence listing part)	: 13
claims	: 02
abstract	: 01
drawings	: 09
sequence listing part of description	:
Total number of sheets	: 29

This international application is **accompanied by** the item(s) marked below:

1. ☐ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☒ other *(specify)*: Request for fax acknowledgement

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: English

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Fabrizio MINOJA

13 February 2001

For receiving Office use only

1. Date of actual receipt of the purported international application: 13 FEB 2001 (13.02.2001)		2. Drawings: ☒ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:		
4. Date of timely receipt of the required corrections under PCT Article 11(2):		
5. International Searching Authority (if two or more are competent): ISA/	6. ☐ Transmittal of search copy delayed until search fee is paid.	

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Date of receipt of the record copy by the International Bureau:

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
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31 January 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/61334 A3

(54) Title: NEW METHODS FOR SURFACE MODIFICATION OF SILICA FOR USE IN CAPILLARY ZONE ELECTROPHORESIS AND CHROMATOGRAPHY

(57) Abstract: The present invention refers to the use of novel molecules able to bind tenaciously to silica, borosilicate and silicate surfaces, and thus to modify their properties and characteristics. When applied to silica-based chromatography, it offers important advantages in all cases in which it is necessary to modulate the interaction of analytes with the stationary phase. In capillary zone electrophoresis (CZE), such compounds will eliminate or invert the electroendoosmostic (EEO) flow, greatly simplifying the analysis of negatively-charged compounds and permitting the analysis of bio(macro)molecules via the direct use of naked capillaries.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/01544

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N27/447

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C07D C08G

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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A	US 2 417 992 A (VICTOR NIEDERL ET AL) 25 March 1947 (1947-03-25) claim 1 --- -/--	5

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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°A° document defining the general state of the art which is not considered to be of particular relevance

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°P° document published prior to the international filing date but later than the priority date claimed

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°X° document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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°&° document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 October 2001	08/11/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Duchatellier, M

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 01/01544

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 01/01544

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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NEW METHODS FOR SURFACE MODIFICATION OF SILICA FOR USE IN CAPILLARY ZONE ELECTROPHORESIS AND CHROMATOGRAPHY

The present invention refers to the use of novel molecules able to bind tenaciously to silica, borosilicate and silicate surfaces, and thus to modify their properties and characteristics. When applied to silica-based chromatography, it offers important advantages in all cases in which it is necessary to modulate the interaction of analytes with the stationary phase. In capillary zone electrophoresis (CZE), such compounds will eliminate or invert the electroendoosmotic (EEO) flow, greatly simplifying the analysis of negatively-charged compounds and permitting the analysis of bio(macro)molecules via the direct use of naked capillaries.

The fused silica is constituted of three types of ionizable silanols: isolated, geminal and vicinal. The density of such groups has been estimated of the order of 5 silanols per nm^2, whose average pK_a value has been estimated as 6.3 (M.S. Bello, L. Capelli e P.G. Righetti, *J. Chromatogr.* A 684, 1994, 311). Thus, at any operative pH value above 2, there will be a fraction of ionized silanols, fraction which will be larger and larger at progressively higher pH values till reaching a plateau at pH ca. 10.

The EEO flow in a fused silica column is produced by the electric field and is transmitted by the drag of ions in a thin liquid layer adjacent to the silica wall. The origin of the net positive charge in this thin liquid sheath is due to the progressive ionization of silanol groups in the wall. The electric potential generated by these fixed negative charges generates a diffuse double layer (called Debye-Hückel layer) in which there exist an excess of cations as compared with anions in the buffer present in solution. When the electric circuit is closed, this excess of cations is continuously perturbed and drugged toward the negative pole (the cathode). Since the cations coordinate a number of hydration water molecules, the continuous

migration of this excess of cations generates a net water transport from the anode to the cathode, called EEO flow. This flux continues as long as the electric field is applied, since the Debye-Hückel double layer is continuously perturbed by the applied potential difference and thus it has to be continuously reformed. The EEO flow in CZE has been studied in depth, due to its fundamental importance in understanding the results of electrophoretic separations e due to its strong influence on the reproducibility of transit times. The reproducibility of the EEO flow is in fact rather modest, particularly in proximity of the pK_a, where the EEO vs. pH curve exhibits the highest slope. This is also due to the slow equilibration of the silica surface in changing from acidic to alkaline solutions, due, for instance, to strongly acidic or basic pH values adopted in washing the silica column after electrophoretic analysis of complex analytes, which could leave material adhering to the wall. This slow equilibration process causes dramatic variations of the EEO flux, which, in turn, could provoke poor reproducibility of the transit times of analytes, both between runs and during different days of analysis.

Per se, the EEO flux is not noxious to the electrophoretic process; on the contrary its presence is of fundamental importance when attempting separation in a single run of mixtures of anionic, cationic and neutral substances. At elevated EEO fluxes, it is possible that even negatively charged analytes, which would normally migrate to the anode, will be transported to the cathode, thus being detected at the monitoring window (in normal polarity runs the cathode is placed close to the detector). The presence of the EEO flux is of fundamental importance in methods such as electrokinetic micellar chromatography (MEKC), in which the analytes are adsorbed onto a surfactant (typically Na dodecyl sulphate, SDS). Since the surfactant micelles migrate towards the anode, but generally with lower velocities as compared with that of the EEO flux, at appropriate pH values, there is a large time window for separating both neutral and hydrophobic analytes which interact to some extent with said micelles. On the contrary, in numerous other cases, the

presence of negative charges on the wall (to which the EEO flux is associated) is strongly detrimental to the electrophoretic separation. One of the most serious problems, in this case, is the adsorption of cationic analytes. Whereas such adsorption, in the case of small molecules, might be of modest entity, reversible and thus provoke only moderate losses of resolution, in the case of macromolecules, especially for proteins and peptides, this phenomenon is disastrous and could cause not only strong peak asymmetry, but even complete loss of analyte when totally and irreversibly adsorbed to the wall. Even in the case of DNA separations such EEO flow is noxious, since it causes peak asymmetry and elution of sieving liquid polymers from the capillary lumen. Over the years, many solutions have been proposed for solving this problem as reviewed in e.g., M. Chiari, M. Nesi e P.G. Righetti, in Capillary Electrophoresis in Analytical Biotechnology, P.G. Righetti, Ed., CRC Press, Boca Raton, 1996, pp. 1-36; F.E. Regnier e S. Lin, in High Performance Capillary Electrophoresis, M.G. Khaledi, Ed., Wiley, New York, 1998, pp. 683-728; G.M. McLaughilin et K.W. Anderson, in High Performance Capillary Electrophoresis, M.G. Khaledi, Ed., Wiley, New York, 1998, pp. 637-681.

Among the various solutions proposed for eliminating the EEO flux, we can recall here:

a) Variations in the type of buffer and its additives;

b) Adsorbed coatings (e.g., neutral polymers, neutral, charged or zwitterionic surfactants);

c) Covalently bound polymers, typically neutral macromolecules, such as acrylamides and celluloses, bound to the wall usually via bifunctional molecules (bridging or cross linking agents).

Covalently bound polymers have been found to be the most effective in quenching EEO flux, not only because the wall should be physically carpeted with neutral polymers, but also because, due to the anchoring of the polymers to the free

silanols, there is an overall suppression of negative charges. However, such coatings are the most expensive among those offered on the market, and cannot be easily performed in individual laboratories, since good skills in organic chemistry and specialized equipment are required. In addition, this type of coating undergoes progressive deterioration during use, which calls for replacement of the capillary, this adding to the costs of analysis.

For all these reasons, dynamic capillary coatings, as obtained by additives to the background electrolyte, have been much preferred and definitely more popular among users. Among the buffer modifications there could be very simple ones, such as changes of the operative pH (e.g., at pH extremes the proteins are either repelled by the capillary, at alkaline pHs, or are not adsorbed, because the wall is neutral, at acidic pHs), or changes in the type of cation, or even the use of hydro-organic solvents, or yet strong changes in the buffer molarity (at high buffer concentrations interactions with the capillary wall are quenched or discouraged).

Each of these modifications can present some advantages, but also a number of disadvantages. A highly promising research line is the one which utilizes oligo-amines (especially tri-, tetra- and penta-amines). Oligo-amines are adsorbed to the wall via cooperative linkages, due to the presence of multiple charges on the skeleton of nitrogens and are thus able to minimize and often complete eliminate protein and peptide adsorption to the wall. Among these classes of compounds, the best ones appear to be spermine (a skeleton of four nitrogens separated by two or three carbon atoms) and TEPA (tetraethylene penta-amine) composed by a skeleton of five nitrogens separated by ethylene groups. This last molecule belongs to a large family of polyazotated compounds, both linear and branched. It would appear that the efficacy of such oligo-aminic compounds increases as a function of molecular mass as well as of the CH_2/NH ratio and of the total number of ethylene groups in the molecule.

Even though the oligo-amines appear to be extremely promising both because

of the ease of their use and for the efficiency of the coating, they present a common, most prominent defect: at neutral and alkaline operative pH values (the latter being the most popular for protein separations) they exhibit a drastic decrease of efficacy, since their nitrogens are progressively deprotonated, this in turn hampering the co-operative linkage to the wall (such linkage being mostly of ionic type).

Also in chromatographic processes utilizing silica beads as supports for covalent linkage of a variety of phases, silanol ionization represents a serious problem. For instance, in reversed-phase (RP)-HPLC, many companies produce silica spheres, to which hydrophobic phases, such a $C_{18}H_{37}$ (C_{18} phases) or C_8H_{17} (C_8 phases), are covalently affixed. Although reactions are carried out under conditions which should ensure full reaction of free silanols, in practice, due to steric hindrance, barely 50% of the silanol population can react (J.C. Dolan, Liquid Chrom. Gas Chrom. Int. 12, 1999, 156; D.V. McCalley, Liquid Chrom. Gas Chrom. Int. 12, 1999, 638; D.C. Leach, M.A. Stadalius, J.S. Berus & L.R. Snyder Liquid Chrom. Gas Chrom. Int. 1, 1988, 22). As a consequence, in the separation of basic compounds, peaks are strongly tailed with loss of resolution, and sometimes even total loss of analyte occurs, due to irreversible adsorption onto the silanolic phase. As a remedy, one has tried to react free silanols (the ones still not bound with C_{18}, C_8 phases etc.) with silanic agents of small size, such as trimethylchloro silane, a procedure called end capping. However, even end-capped phases still present ½ of the silanols unreacted, which means that the problems is lessened but not abolished. In order to further minimize this problem, in silica-based chromatography, already in the seventies, additives to the eluent have been proposed, able to block ionized silanols via salt bridges. Among those additives, the most popular one is triethylamine, at concentration 20-50 mM. The compounds described in the present invention, being able to bind to free silanols, are highly efficient in ameliorating chromatographic separation, as described below.

The present invention describes a novel class of molecules able to overcome

all the drawbacks described above.

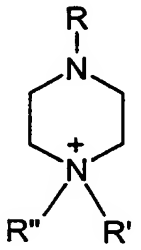
The compounds here claimed possess the following structural characteristics:

a) the presence of one or more quaternary nitrogens able to form ionic bonds with silanols at any operative pH value;

b) the presence of one or more basic atoms (*tertiary* nitrogen or oxygen, either ethereal or carbonyl) able to form hydrogen bonds or electrostatic interactions via the same heteroatoms at different pH values along the pH scale;

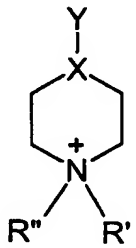
c) the presence of one or more alkyl chains (typically but not exclusively C-4), possessing terminal carbon atoms substituted with one or more electronegative atoms able to react with silanolic groups to such an extent as to form covalent bonds with the capillary wall.

Particularly effective appear to be quaternary ammonium salts derivatives possessing the structural formula **1, 2** and **3**.



1

2



3

where R represents typically (but not exclusively) a CH_3, whereas R' and R", independently-between them, represent typically (but not exclusively) either a CH_3, or a group with formula $-[(CH_2)_n]-Z$, where n = 2 o > 2, preferably 4, and Z = halogen, OH, O-alkyl (with 1-4 carbon atoms), $O-SO_2C_6H_5CH_3$, N_3. Compounds of relevant interest are also the heterocyclic derivatives of formula **3**, where X=O, Y≠; or X = C, Y = O; or X=CH, Y=OR'''; or X=CH, Y=H, alkyl (C_1-C_{10}).

The preferred substituents in compounds of formula **1, 2** and **3** are R = CH_3, R' = CH_3, R" = $(CH_2)_4I$.

Other compounds covered by the present invention are molecules of type

above indicated where the heterocyclic ring and/or the alkyl residues contain one or more asymmetric carbons so as to be utilized as chiral selectors.

The compounds described in the present invention can be utilized either as additives to the background electrolyte (typically at acidic pH values), or as wall modifiers introduced only during the pre-conditioning phase of the capillary (typically at neutral or alkaline pH values). Under the latter conditions, given the absence of the modifier in the background electrolyte, one obtains the unique advantage of ameliorating the signal to noise ratio, thus improving *post-column* techniques for analyte detection.

According to the invention described so far, the synthesis of one of these families of compounds and examples on separations of both small organic molecules and macromolecules are here illustrated.

Synthesis of a derivative type 1 with R=CH_3, R' = CH_3 and R'' = -$(CH_2)_4I$ (QPzI)

11.4 g of N,N'-dimethylpiperazine (0.1 mol) are dissolved in 100 mL of diethylether. This solution, under stirring, is added with a mixture of 31 g 1,4-diiodobutane (0.1 mol) in 100 mL ether and let to react for 12 h. The precipitate formed (38.2 g) is filtered, washed with diethylether and dried with a vacuum pump.
H-NMR (DMSO) δ (ppm): 1.71-1.88 (m, H4), 2.28 (s, 3H), 2.58-2.68 (m, H2), 2.68-2.78 (m, 2H), 3.05 (s, 3H), 3.3 (t, 2H), 3.35-3.45 (m, 6H).
MS(MALDI): 296 (M+-I, 100), 169 (28). F.f. 278-280°C.

Example 1

The analysis of carboxylic acids containing heterocyclic rings with nitrogen groups, when performed in an uncoated capillary, is besieged by problems due to adsorption of such molecules to the capillary wall. The analysis of dilute solutions of such compounds is thus impossible, unless properly coated capillaries are adopted.

Figure 1 reports the electropherogram pertaining to the analysis of a solution

of picolinic acid, at 1 mM concentration, in an uncoated capillary. The wall adsorption clearly originates a broad peak, with very poor plate count.

Figure 2 gives the analysis of a mixture of picolinic and nicotinic acids at 50 micromolar concentration, performed with the procedure of wall modification described in the present invention. In **Figure 2**, it is evident how the brief capillary pre-treatment with the modifier QPzI effectively abolishes the analyte adsorption to the capillary wall, allowing thus proper quantitation of species present even at low levels, as often required in the analysis of biological fluids, in environmental analysis and in the food industry.

When operating with a new capillary, it is necessary to perform a brief pre-conditioning of the capillary, consisting in a few washing cycles, as described below, till reaching constant EEO flux values or, if required, inversion of the EEO flux.

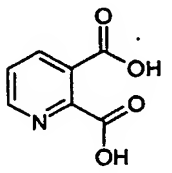
Pre-conditioning: washing (5 bar for 2 min) with a modifier solution (2-4 mM in borate buffer, 25 mM, at pH 9.0), followed by a brief washing (5 bar for 4 min) with running buffer.

Sample analysis is performed according to the following **procedure 1**: washing (5 bar for 2 min) with a appropriate modifier solution (2-4 mM in borate buffer, 25 mM, at pH 9.0), followed by a washing (5 bar per 4 min) with running buffer, sample injection (10-20 mbar for 10 s), injection of a running buffer plug (5 mbar for 5 s).

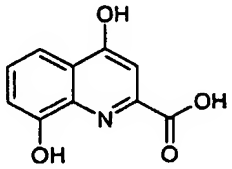
Example 2

Separation of a series of tryptophan metabolites

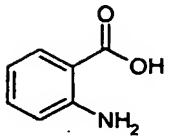
The formulae of the various analytes are the following:



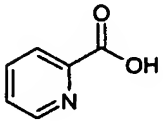
Quinolinic acid **1**



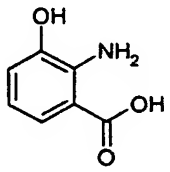
Xanthurenic acid **2**



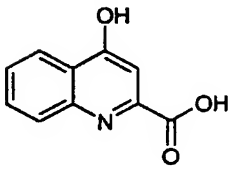
Anthranylic acid **3**



Picolinic acid **4**



3-Hydroxyanthranylic acid **5**



Chinurenic acid **6**

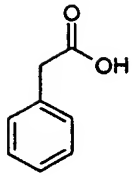
Figure 3 shows a separation obtained in CZE with the modifier QPzI as covered in the present invention. The analysis of the mixture is performed according to **procedure 1**, thus in the absence of the modifier in the running buffer. One should note the excellent separation of all six compounds in the mixture, coupled to very short analysis times (<6 min). It has not been possible to obtain such results in an uncoated capillary both due to unfavorable EEO flow and to adsorption of analytes.

Also the use of conventional oligoamines (spermine and TEPA) has not given any appreciable result. In this particular case the EEO is inverted, due to an excess of positive charges present in the piperazine bound to the silica wall. This flux inversion has been verified via the elution of a neutral marker (compound No. 7: acrylamide), eluted in ca. 10 min

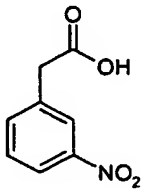
Example 3

Separation of arylalkanoic acids

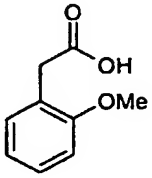
The formulae of the various analytes are the following:



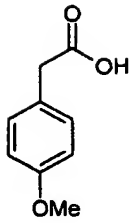
Phenylacetic Ac. **1**



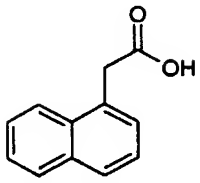
3-NO_2-phenylacetic Ac. **2**



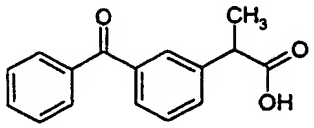
2-OMe-phenyl-acetic Ac. **3**



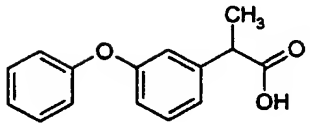
4-OMe-phenyla-cetic acid **4**



1-Naphthylacetic Ac. **5**



2-(3-benzoylphenyl)propionic acid **6** (Ketoprofen)



2-(3-phenoxyphenyl)-pro-pionic acid **7** (Fenoprofen)

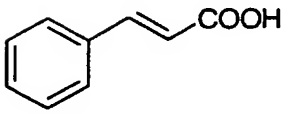
Figure 4 shows the electropherogram of a mixture of seven alkanoic acids analyzed with the modifier QPzI according to **procedure 1**.

Also in this case no separation has been possible with either coated capillaries or with background electrolytes containing conventional oligo-amines (e.g., spermine, TEPA), alone or in a mixture. It is believed, in fact, that the separation of **Figure 4** has been made possible by the interaction of the analytes with the piperazine adsorbed to the wall. In this case, thus, the piperazine acts by both modulating (or inverting) the EEO flux and by becoming an active player in the separation process, due to its interaction with some analytes.

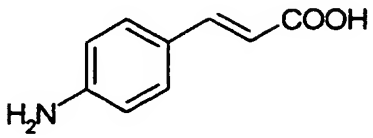
Example 4

Separation of cinnamic acids

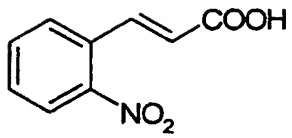
The formulae of the various analytes are the following:



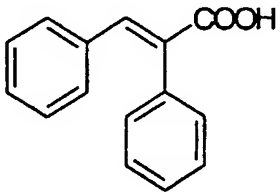
trans-cinnamic acid **1**



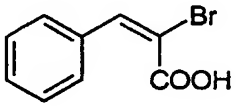
trans-p-aminocinnamic acid **2**



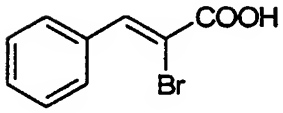
trans-o-nitrocinnamic acid **3**



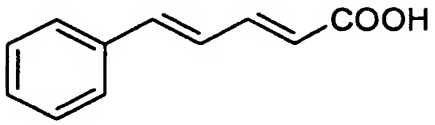
trans–α–phenylcinnamic acid **4**



trans–α–bromocinnamic acid **5**



cis-α-bromocinnamic acid **6**



trans-5-phenylpentadienoic acid **7**

Figure 5A shows the electropherogram of a mixture of seven cinnamic acids analyzed with an untreated fused silica capillary. It is evident the poor separation of the compound 2, 3, 5 and 6 that, on the contrary, can be well resolved using a capillary treated with the modifier type 3 (X=C, Y=O, R'=CH_3 and R''=$(CH_2)_8I$ in according with the procedure 1 **(Figure 5B)**.

Example 5

Separation of proteins at alkaline pH

These separations are of great interest, since at this operative pH (pH 9.0) proteins are kept in a native state. **Figure 6** shows the profiles of a five different proteins, injected in a covalently-coated vs. a QPzI-treated capillary, respectively. In

the case of protein separations performed in covalently-coated capillaries, the running time are longer than observed in QPzI-treated capillaries and the peaks are broad, possibly due to diffusion, whereas the peak areas remain substantially the same, indicating the absence, or at least the same sample adsorption, to the wall.

The separation of a mixture of protein with pI ranging from pH 3 to pH 10 is shown in **Figure 7**. It can be appreciated that the separation occurs according to the protein mobility and EOF. The first protein eluted is thus the moderately alkaline horse myoglobin acidic and basic band, then the acidic one which the mobility is influenced by the positive charge docked into the wall. The last group of protein is represented by the most alkaline ones, first the lentil lectins with pIs ranging from pH 8.15 to 8.65, just before the neutral marker, and then trypsinogen (pI 9.5) which overlaps with the neutral acrylamide marker. Peak identification was performed by direct spotting of the pure protein into the calibration pI mixture. **Figure 7** shows the same protein mixture injected in a covalently coated capillary (left); in this case a single peak representing the 9 proteins is obtained, indicating that interactions between the modified wall and proteins are important not only in avoiding absorption but in playing an active in the separation process.

Example 6

Separation of proteins at acidic pH

These separations are of interest because, in principle, they can be performed in uncoated capillary, in the absence of any modifier, due to the absence of ionization of silanols at pH values of ca. 2.0-2.5.

However, at such strongly acidic pH values, it is to be expected that proteins will be denatured and will loose their tridimensional structure. Thus, it is better to ensure that this process will be brought to completion and therefore such analyses are typically performed in presence of denaturing/solubilizing agents, such 8 M urea. In turn, in 8 M urea solutions, the apparent pH value of the solution increases by about 1 pH unit, thus rendering quite real the risk of adsorption of proteins and

peptides to the silica wall. **Figure 8** shows the ability of various additives in inhibiting protein adsorption to the wall (a mixture of α e β human globin chains) in an amphoteric, isoelectric buffer composed of 50 mM imino diacetic acid (IDA). The pH of this IDA solution is of 2.3 (pH=pI), but the addition of 8 M urea (necessary for keeping in solution the globin chains) raises the apparent pH value to ca. 3.2. It is seen how various additives, either alone or in combination, have inhibition powers ranging from ca. 95 to 98%. Only the presence of QPzI in solution (1 mM) is able to reduce protein adsorption to barely 0.5%.

In all these separations, the dynamic modifiers must always be present in solution, even in the background electrolyte during the run. This does not apply to the piperazine modifier if the capillary is first conditioned at alkaline pH values (pH 9.0) and then equilibrated in the low pH buffer. At higher pH values, in isoelectric Asp buffer (apparent pH 3.8 in 8 M urea) the situation readily deteriorates: in presence of some additives, the amount of globin chains bound to the wall reaches extremely high values, as high as 45% of the initial, injected amount. Only in presence of 1 mM piperazine alone this value is reduced to only 3% **(Figure 9)**. It is thus demonstrated that the piperazine additive not only can be used very efficiently along the entire pH scale for electrophoretic separations, but also that it is by far the most efficient additive in inhibiting interaction and adsorption of macromolecules to the silica wall.

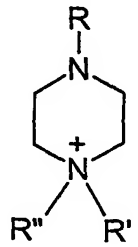
CLAIMS

1. Compounds able to modify silica surfaces and/or to inhibit or inverting the EEO flow in capillary electrophoretic separations, characterized by the following functional groups: a) one or more *quaternary* nitrogens; b) one or more basic atoms; c) one or more C_2-C_5 alkyl chains containing at the end a carbon atom substituted with one ore more electronegative atoms,

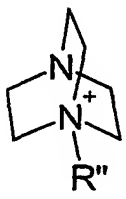
said compounds optionally containing asymmetry centers.

2. Compounds as claimed in claim 1, wherein the basic atoms according to b) are selected from the group consisting of *tertiary* nitrogen or oxygen, either ethereal or carbonyl, and the alkyl chains according to c) are C_4-alkyl chains.

3. Compounds as claimed in claims 1 and 2, of formula **1** and **2**,



1

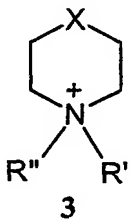


2

wherein R is a C_1-C_4 alkyl group, and R' and R'' are independently a (C_1-C_4) alkyl group or a group of formula $[(CH_2)_n]Z$, where n = 3-6 and Z is halogen, hydroxy, (C_1-C_4) alkoxy, p-toluenesulphonyloxy or N_3.

4. A compound of formula **1** as claimed in claim 3, wherein R and R' are CH_3 and R'' is $-(CH_2)_4$-I.

5. Compounds of formula **3**



3

wherein X is O, CO, CH_2, or CH-(C_1-C_{10}) alkyl; R is (C_1-C_4) alkyl and R' and R''

are independently (C_1-C_4) alkyl or a group of formula [$(CH_2)_n$]-Z, wherein n is 3-6 and Z is halogen, hydroxy, (C_1-C_4) alkoxy, p-toluenesulphonyloxy or N_3.

6. Compounds as claimed in claim 5, wherein R and R' are CH_3 and n is 4.

7. The use of the compounds as claimed in claims 1 to 6 for chromatographic separations utilizing silica-based material.

8. The use of spheres and of silica material in general, treated with the compounds as claimed in claims 1 to 6, for chiral chromatographic separations.

9. The use of the compounds as claimed in claims 1 to 6 for coating glass and borosilicate surfaces as used in nanotechnologies for electrophoretic separations of any class of molecules.

10. The use as claimed in claim 9, for coating chips as used in hyphenated techniques, chips interfaced with chromatographic columns, with mass detectors and other separation / detection devices, including two-dimensional separation methods.

11. The use of capillaries treated with the compounds as claimed in claims 1 to 6 for separations of proteins and peptides, at any value of the pH scale necessary for optimizing such separations, including capillary electrophoresis using hyphenated techniques.

12. The use of capillaries treated with the compounds as claimed in claims 1 to 6 for separations of proteins and peptides in both conventional buffers and amphoteric, isoelectric buffers, either acidic or neutral or alkaline.

13. The use of capillaries treated with the compounds as claimed in claims 3 to 6 for separations of oligonucleotides and DNA fragments, in both conventional buffers and amphoteric, isoelectric buffers, either acidic or neutral or alkaline.

14. The use of capillaries treated with the compounds as claimed in claim 3 to 6 for separations of small molecules able to interact with the capillary wall or whose separations might be hampered by the EEO flow of non-conditioned capillaries.

15. The use of capillaries treated with the compounds as claimed in claims 3 and 5 for chiral separations.

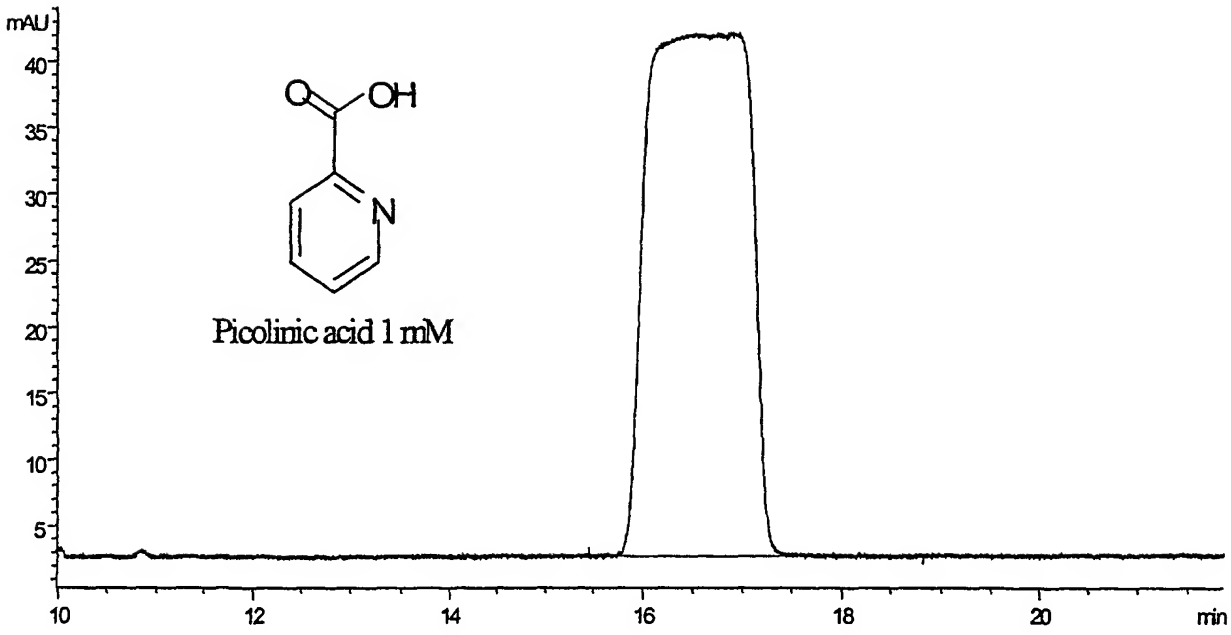


Figure 1: analysis conditions: fused silica capillary ϕ = 50 µm, $L_{tot.}$ = 60 cm, 50 mM borate buffer, pH = 9.0, + 15 kV, T = 20°C, λ = 210 nm.

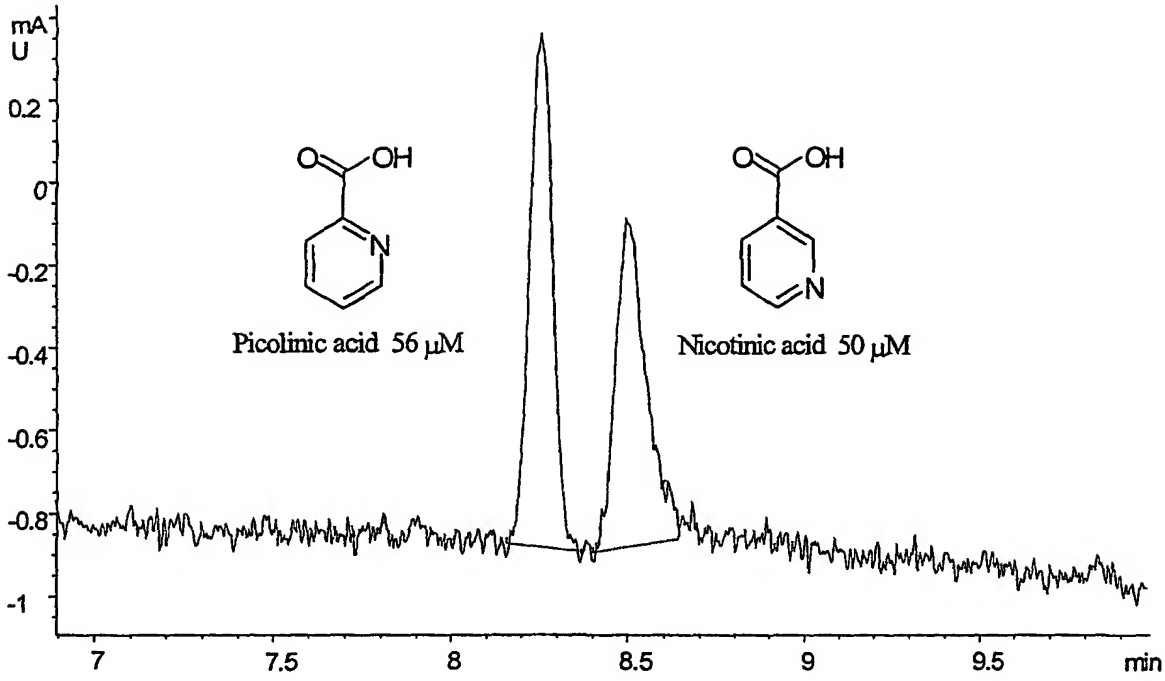


Figure 2: analysis conditions: fused silica capillary, pre-treated for 5 min with a 1 mM solution of compound (1), $\phi = 50$ µm, $L_{tot.} = 60$ cm, 25 mM borate buffer, pH = 9.0, -20 kV, T = 20°C, $\lambda = 210$ nm.

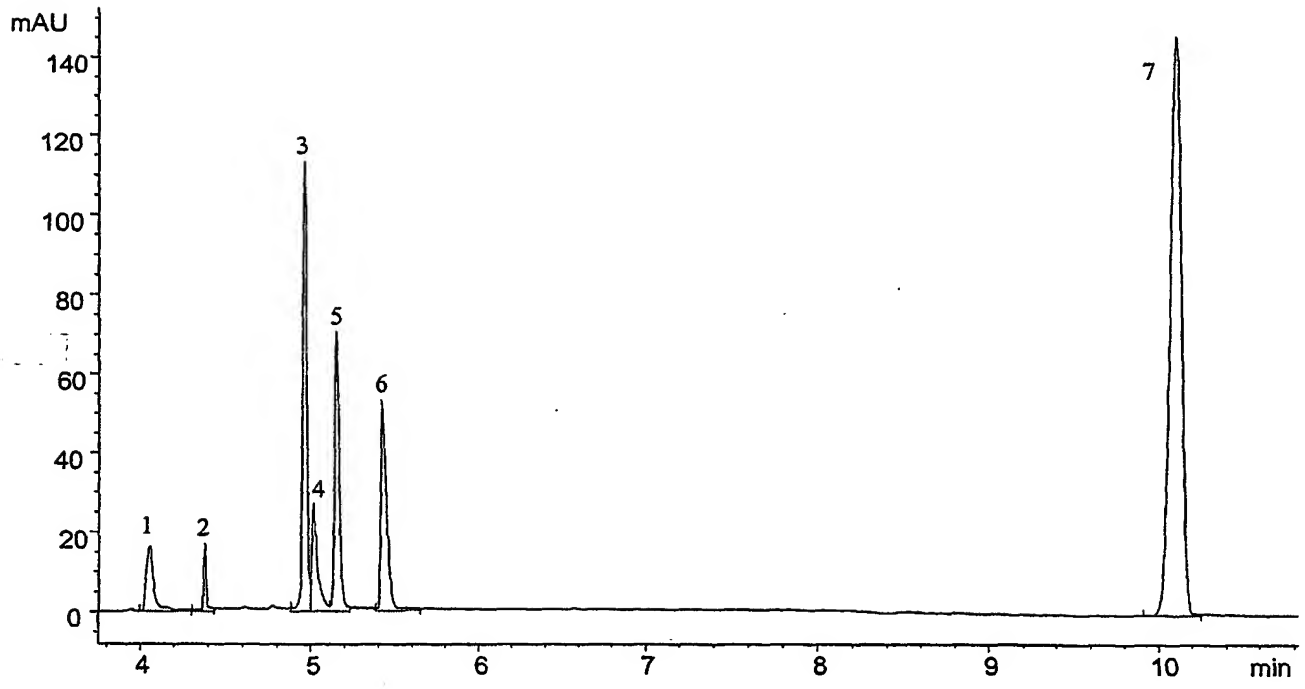


Figure 3: analysis conditions: fused silica capillary, $\phi = 50$ µm, $L_{tot.} = 60$ cm, 25 mM borate buffer, pH = 8.5, - 20 kV, T = 20°C, $\lambda = 210$ nm. Analyte concentration: 0.2 mg/ml.

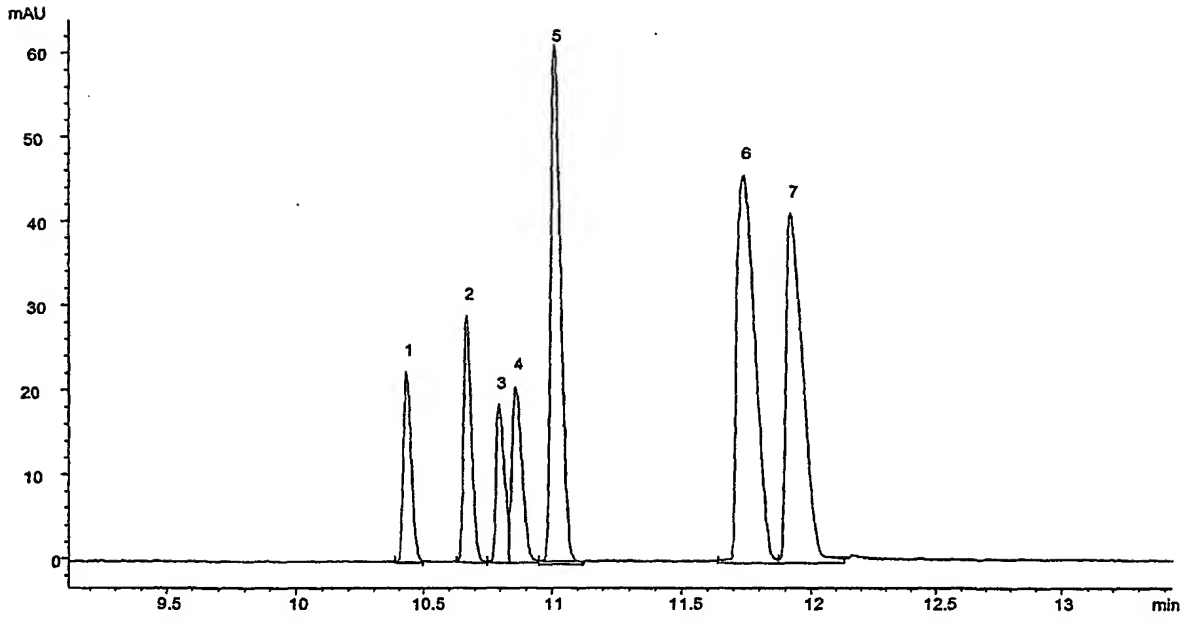
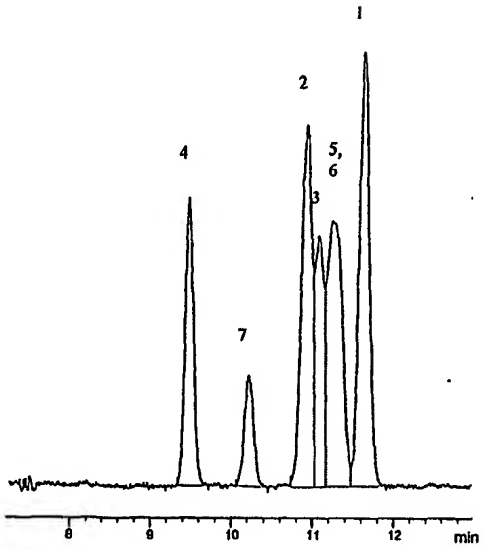


Figure 4: analysis conditions: fused silica capillary ϕ = 50 µm, $L_{tot.}$ = 100 cm, 25 mM borate buffer, pH = 8.5, - 25 kV, T = 25°C, λ = 210 nm. Analyte concentration: 0.14 mg/ml.



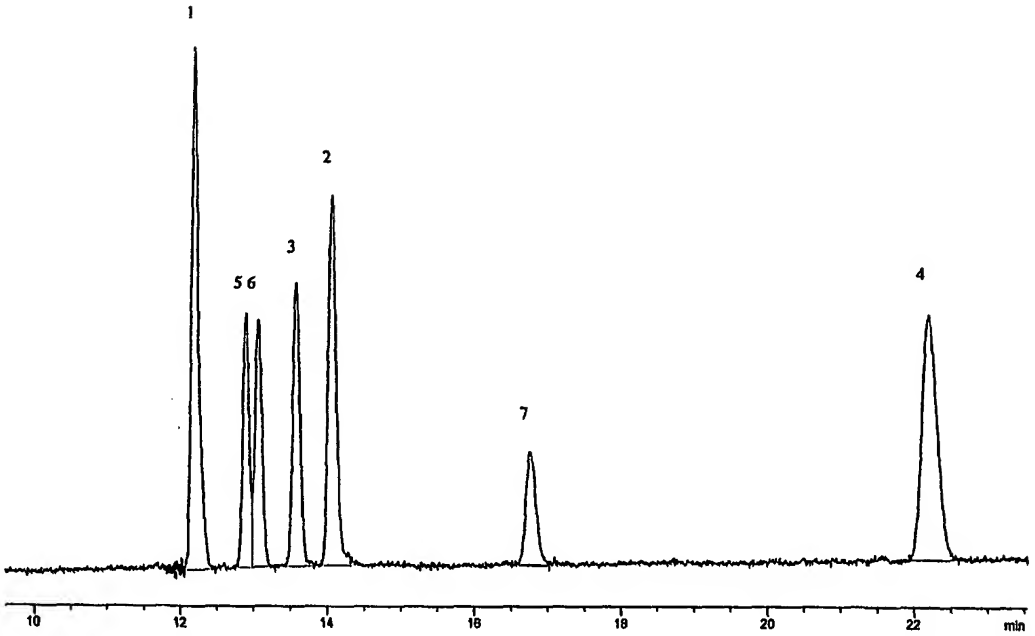


Figure 5A: analysis conditions: uncoated fused silica capillary $\phi = 50$ µm, $L_{tot.} = 50$ cm, 25 mM borate buffer, pH = 9, + 15 kV, T = 25°C, $\lambda = 210$ nm.i

Figure 5B: analysis conditions: fused silica capillary $\phi = 50$ µm, $L_{tot.} = 50$ cm, 25 mM borate buffer, pH = 9, - 25 kV, T = 25°C, $\lambda = 210$ nm.

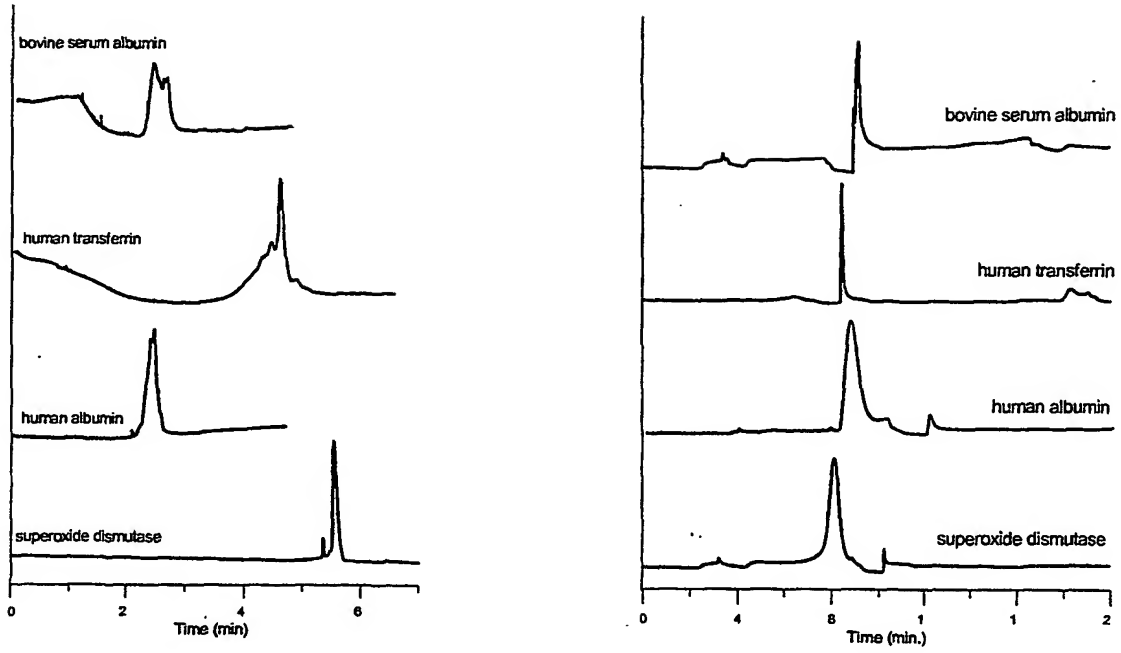


Figure 6: Separation of a number of protein markers, injected in a covalently coated (left) and in a **Q-PzI** treated (right) capillary, respectively. Capillary length 37 cm, 50 µm i.d.. Separation conditions were: run at 200 V/cm, sample injection by pressure for 2 sec, 5 psi/s, detection at 214 nm. In both cases the running buffer was 25 Mm Na tetraborate, Ph 9.0.

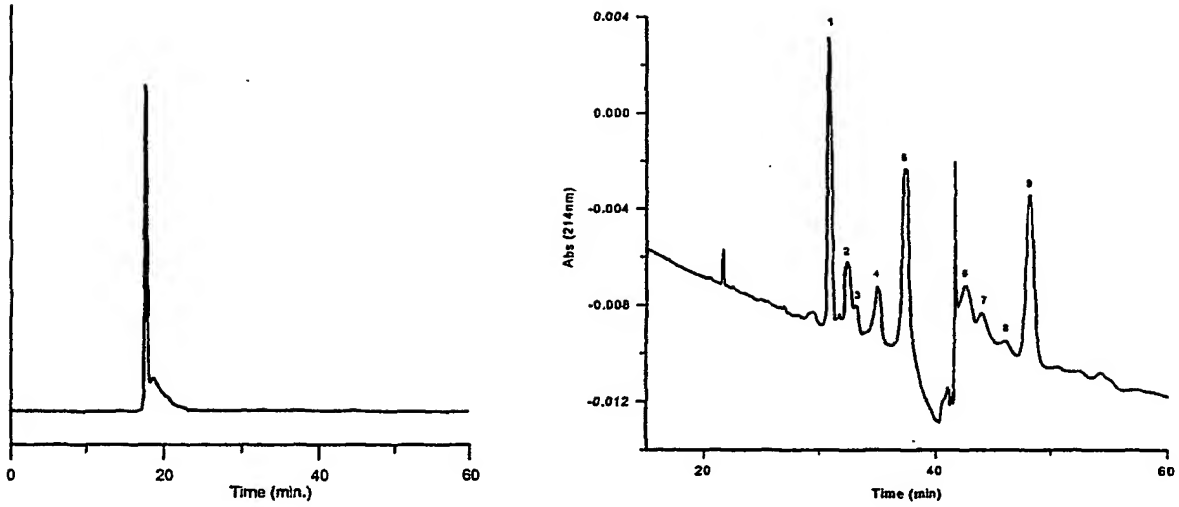


Figure 7: Separation of protein mixture with Pi ranging from Ph 3-10 (right) with **QpzI** treated capillary 77 cm long, 50 μm i.d.; (left) covalently coated capillary , 77 cm long, 50μ m, i.d..

Separation conditions: 250V/cm, sample injection by pressure for 5 sec, running in tetraborate buffer Ph 9.0.(1) Horse myoglobin, (2) bovine carbonicanhydrase B, (3) human carbonicanhydrase B, (4) β-lactoglobulin A, (5) soybean trypsin inhibitor, (6) lentil-lectin Pi 8.15 (7) lentil-lectin Pi 8.55, (8) lentil-lectin Pi 8.65, (9) trypsinogen

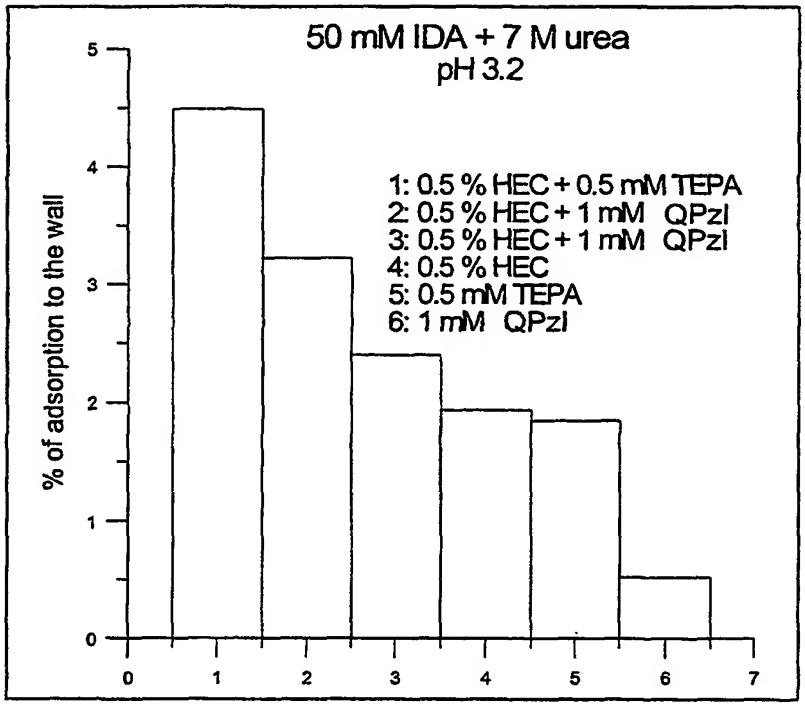


Figure 8: inhibition ability of different additives to the binding of proteins to the silica wall. The electrophoretic runs have been performed in 50 Mm IDA buffer, in presence of 8 M urea (apparent Ph of 3.2) in Waters Quanta 4000E instrument, in a 27-cm-long uncoated capillary, 50 μm ID. Sample: mixture of α and β human globin chains, 2 mg/Ml. After 10 consecutive runs, the adsorbed proteins are eluted electrophoretically in 25 Mm phosphate buffer, Ph 7, containing 60 Mm SDS and detected at 210 nm.

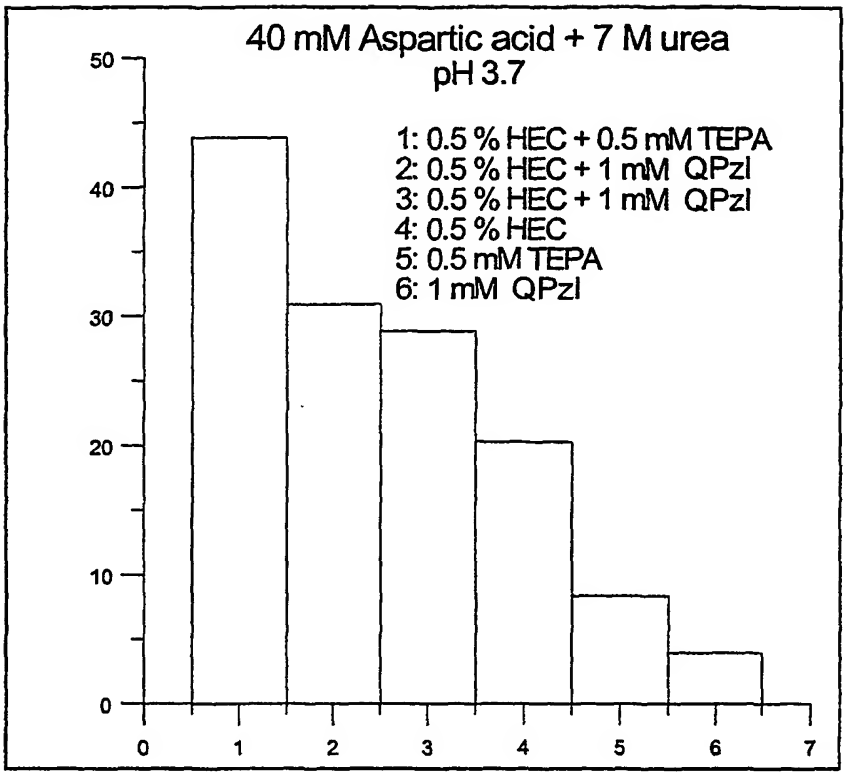


Figure 9: inhibition capability of various additives toward the adsorption of proteins to the silica wall. The electrophoretic runs have been executed in 50 mM Asp buffer in presence of 8 M urea (apparent pH 3.8) in a Waters Quanta 4000E instrument, in 27-cm-long, uncoated capillary, 50 μm ID. Buffer: a mixture of α e β human globin chains, 2 mg/mL. After 10 consecutive runs, the adsorbed proteins are eluted electrophoretically in 25 mM phosphate buffer, pH 7, containing 60 mM SDS and detected at 210 nm.

PATENT COOPERATION TREATY

REC'D 13 MAY 2002
WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

10/069567

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
SCB611PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP01/01544	13/02/2001	18/02/2000
International Patent Classification (IPC) or national classification and IPC G01N27/447		
Applicant CITTERIO, Attilio et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
10/09/2001	10.05.2002
Name and mailing address of the international preliminary examining authority: European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Authorized officer Duchatellier, M Telephone No. +31 70 340 2272

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP01/01544

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-13	as originally filed				

Claims, No.:

1-8	as received on	16/04/2002	with letter of	15/04/2002	

Drawings, sheets:

1/9-9/9	as originally filed				

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-8
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-4, 7, 8
	No:	Claims	5, 6
Industrial applicability (IA)	Yes:	Claims	1-8
	No:	Claims	

2. Citations and explanations
   see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1). Reference is made to the following documents:

D1: US-A-5391274

D2: DE-C-836937

D3: US-A-4904629

D4: US-A-2417992

D5: US-A-4014678

D6: US-A-3366638

D7*: US-A-4690749

*This document was not cited in the search report.

2). The invention relates to compounds for use as covalent coating agents for silica-based chromatography.

Such compounds are known from D7 (see claim 1).

The problem to be solved is to obtain an improved modification of the electro-endosmotic flow.

This problem is solved by compounds having the following functional groups: a) one or more quaternary nitrogens; b) one or more basic atoms; c) one or more C_2-C_5 alkyl chains containing at the end a carbon atom substituted with one or more electro-negative atoms.

No available document recites this solution, and this solution cannot be considered as obvious by the skilled man. Consequently, the subject-matter of claim 1

fulfills the requirements of Article 33(3) PCT.

3). Dependent compound-claims 2-4.

The dependent claims 2-4 are truly dependent claims which relate to further embodiments of the subject-matter of claim 1 or of another claim dependent therefrom and therefore also fulfill the requirements of Article 33 (3)-(5) PCT.

4). Independent compound-claim 5.

4.1 The scope of claim 5 does not correspond to the scope of claim 1. Consequently, a problem of non-unity arises.

4.2 Compound 3 is known from D4 for X=O (cf. column 2, lines 17-20), from D5 for X=CH and Y=H (see abstract) and from D6 for X=C and Y=O (cf. column 2, lines 25-30). In this last case the nitrogen is not a quaternary one, but it is well known that every trisubstituted nitrogen can be quarternarized.

Some other compounds are possible, but in the description no particular compound is disclosed which could be considered as inventive.

5). Dependent compound-claim 6.

5.1 The scope of claim 6 is not understandable as there is no "R" in the compound of claim 5.

5.2 The compound of claim 5 with R'=CH_3 and n=4 does not seem inventive (see also point 4.2, above).

6). Independent method-claim 7.

As the subject-matter of claim 1 is inventive, the use of the compounds of claim 1 for inhibiting the EEO flow is inventive.

Remark: the expression "chip based technology" neither appears nor is suggested in the description. This expression should not have been added.

7). Dependent method-claim 8.

The dependent claim 8 is a truly dependent claim which relates to further embodiments of the subject-matter of claim 7 and therefore also fulfills the requirements of Article 33 (3)-(5) PCT.

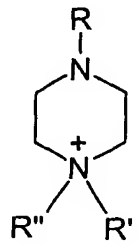
CLAIMS

2. Compounds, characterized by the following functional groups: a) one or more *quaternary* nitrogens; b) one or more basic atoms; c) one or more C_2-C_5 alkyl chains containing at the end a carbon atom substituted with one ore more electronegative atoms,

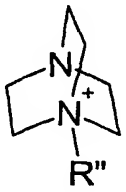
said compounds optionally containing asymmetry centers, for use as covalent coating agents of stationary phases for silica-based chromatography.

2. Compounds as claimed in claim 1, wherein the basic atoms according to b) are selected from the group consisting of *tertiary* nitrogen or oxygen, either ethereal or carbonyl, and the alkyl chains according to c) are C_4-alkyl chains.

3. Compounds as claimed in claims 1 and 2, of formula **1** and **2**,



1

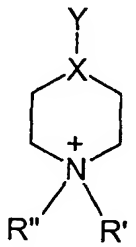


2

wherein R is a C_1-C_4 alkyl group, and independently R' is a (C_1-C_4) alkyl group and R'' is a group of formula $[(CH_2)_n]Z$, where n= 3-6 and Z is halogen, hydroxy, (C_1-C_4) alkoxy, p-toluenesulphonyloxy or N_3.

4. A compound of formula **1** as claimed in claim 3, wherein R and R' are CH_3 and R'' is $-(CH_2)_4$-I.

5. Compounds of formula **3**



3

wherein X=O, Y≠; or X = C, Y = O; or X=CH, Y=OR'''; or X=CH, Y=H, alkyl (C_1-C_{10}), R' is a (C_1-C_4) alkyl group and R'' is a group of formula $[(CH_2)_n]Z$, where n= 3-6 and Z is halogen, hydroxy, (C_1-C_4) alkoxy, p-toluenesulphonyloxy or N_3.

6. Compounds as claimed in claim 5, wherein R and R' are CH_3 and n is 4.

7. The use of the compounds of claims 1-6 for inhibiting or inverting the EEO flow in capillary silica-based chromatography and chip based technologies.

8. The use according to claim 7 for the electrophorectic separation of: oligonucleotides, DNA fragments, peptides or proteins in conventional, amphoteric or isoelectric buffers, either acidic, neutral or alkaline; small molecules interacting with the capillary wall or whose separations might be hampered by the EEO flow of non-conditioned capillaries capillary electrophoresis; chiral compounds.